Unclassified

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REPORT DOCUMENTATION PAGE

AD-A217 764

	1b RESTRICTIVE MARKINGS: N/A
2b DECLASSIFICATION / DOWNGRADING SCHEDULE: N/A	3 DISTRIBUTION / AVAILABILITY OF REPORT: Unlimited
4 PERFORMING ORGANIZATION REPORT NUMBER(S): N/A	5 MONITORING ORGANIZATION REPORT NUMBER(S): N/A
6a NAME OF PERFORMING ORGANIZATION: Dalhousie University; 6b OFFICE SYMBOL (If applicable): N/A	7a NAME OF MONITORING ORGANIZATION: Office of Naval Research
6c ADDRESS (City, State, and ZIP Code): Vice-President, Research Arts & Administration Bldg Halifax, Nova Scotia B3H 4H7	7b ADDRESS (City, State, and ZIP Code): 800 N. Quincy Street Arlington, Virginia 22217-5000
8a NAME OF FUNDING / SPONSORING ORGANIZATION: Office of Naval Research; 8b OFFICE SYMBOL (If applicable): ONR	9 PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: N00014-88-J-1030
8c ADDRESS (City, State, and ZIP Code): 800 N. Quincy Street Arlington, Virginia 22217-5000	10 SOURCE OF FUNDING NUMBERS: PROGRAM ELEMENT NO; PROJECT NO; TASK NO; WORK UNIT ACCESSION

DTIC ELECTE FEB 08 1990 S D Co E

11 TITLE (Include Security Classification)
Development of shuttle vectors for halobacteria

12 PERSONAL AUTHOR(S)
Doolittle, W. Ford

13a TYPE OF REPORT	13b TIME COVERED	14 DATE OF REPORT (Year, Month, Day)	15 PAGE COUNT
Annual	FROM Feb. 1/88 TO Feb. 1/90	February 1, 1990	7

16 SUPPLEMENTARY NOTATION

17 COSATI CODES		
FIELD	GROUP	SUB-GROUP

18 SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
Archaebacteria/halobacteria/plasmid/shuttle vector/ mevinolin resistance, microbiology, genetic engineering and molecular biology

19 ABSTRACT (Continue on reverse if necessary and identify by block number)

We continue to work to develop tools for genetic analysis in the archaebacterium Halofera (Halobacterium volcanii). This includes (i) development of transformation systems, (ii) construction and tailoring of shuttle vectors, (iii) genetic reconstruction of host strain to reduce residual problems associated with restriction and recombination, and (iv) application to a variety of genetic analyses. Among such applications are detailed characterization of the halobacterial HMGCoA reductase gene and of clusters of genes involved in the biosynthesis of tryptophan, histidine, arginine and leucine. Our techniques have allowed construction of a genetic map with several dozen markers, and will permit detailed study of archaebacterial gene structure and function.

20 DISTRIBUTION / AVAILABILITY OF ABSTRACT	21 ABSTRACT SECURITY CLASSIFICATION
☐ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT ☐ DTIC USERS	(U)

22a NAME OF RESPONSIBLE INDIVIDUAL	22b TELEPHONE (Include Area Code)	22c OFFICE SYMBOL
Dr. M. Marron	(202) 696-4760	ONR

DD FORM 1473, 84 MAR — 83 APR edition may be used until exhausted. All other editions are obsolete

SECURITY CLASSIFICATION OF THIS PAGE

ANNUAL PROGRESS REPORT

DATE: 1 FEBRUARY 1990

CONTRACT N00014-88-J-1030

R&T CODE #2441D014

PRINCIPAL INVESTIGATOR: W. Ford Doolittle

CONTRACTOR: Dalhousie University
Halifax, Nova Scotia

CONTRACT TITLE: Development of Shuttle Vectors for Halobacteria

START DATE: 1 February 1988

PERIOD OF PERFORMANCE: 1 February 1988 - 1 February 1990

RESEARCH OBJECTIVE: To develop transformation systems for halophilic archaebacteria, construct a variety of useful halobacterial plasmid vectors including halobacteria - *E. coli* shuttle vectors, and construct suitable halobacterial host strains. We then wish to demonstrate the utility of these genetic tools with several model applications.

PROGRESS (YEAR 2):

During our first year we were able to accomplish many of the basic goals of our contract, in (i) developing and refining a highly efficient transformation system for halobacteria, (ii) developing selectable *Haloferax volcanii* - *Escherichia coli* shuttle vectors, and (iii) developing genetically useful halobacterial hosts for transformation. In our second year we have gone on to make further refinements in these areas and to use these genetic tools productively in exploring the genetics and biochemistry of halophilic archaebacteria. For the sake of coherence, we will place progress in the second year in the context of work done during the first year.

Transformation

At the time of our initial application no useful or reproducible transformation system existed for any of the archaebacteria. In earlier work (84) we had developed an efficient transfection system (~10^7 transfectants per microgram of phage ΦH DNA) for *Halobacterium halobium*. By the end of the first year of our contract, we had used knowledge gained with *H. halobium* transfection to develop transformation methods for *Haloferax volcanii*. (95). Now we can expect spheroplasted cells to take up DNA with great efficiency and to recover well from spheroplasting (> 50% regeneration) so that we routinely find, for example, 10^8 transformants per microgram using the endogenous plasmid pHV2 or its derivatives. We have so far demonstrated efficient transformation with (i) pHV2, and deletions of it, scoring for transformants by blot hybridization (89,95), (ii) pHV2 into which we have shotgun-cloned fragments of wild-type *H. volcanii* DNA involved in various biosynthetic pathways or mevinolin resistance, selecting for protrophic transformants of auxotrophic mutants (98,104) (iii) linear fragments of *H. volcanii* genomic DNA (99), (iv) *H. volcanii* DNA cloned into *E. coli* plasmids and replicated in *E. coli* -- including cosmids from the minimally overlapping set of cosmid clones that is the basis of a nearly complete "bottom-up" map we are preparing -- selecting for prototrophic transformants of auxotrophic mutants (98,102), (iv) restriction fragments of cosmid inserts extracted directly from gels (102), and (v) our shuttle vectors pWL101 and pWL102 (see below) which can be grown and selected in *H. volcanii* on the basis of mevinolin resistance and in *E. coli* on the basis of ampicillin resistance (98). *H. volcanii* possess a restriction system which reduces the efficiency of transformation by a factor of about 10^4 to 10^5 for unmodified DNAs, but in general the efficiency of our

transformation system has been such that restriction has not posed a serious problem. For instance we can obtain hundreds to thousands of prototrophic transformants with tens of nanogram amounts of small gel-purified restriction fragments of *E.coli* -replicated cosmids under conditions where control (mock transfomed) plates show no colonies.

Based on experiments with high molecular weight linear DNA, we believe that homologous recombination in *H. volcanii* is a relatively efficient process. Although transformation with genomic DNA declines rapidly with DNA size below ~25 kb, we have been able to demonstrate transformation with fragments as small as 600 bp (99,102). Thus, it is possible to transform auxotrophs to prototrophy with less-than-gene-size pieces of DNA. We have also shown that single stranded, as well as double stranded, DNAs are capable of transforming spheroplasted *H. volcanii*.

Protocols have been developed for transforming stored frozen cells and for microtiter plate-based transformations (95,99). Both have proved extremely useful in transforming large numbers of strains, and in speeding genetic analyses.

Vector development

Beginning with pHV2, an endogenous 6.4 kb plasmid of *H. volcanii* which we sequenced, we have developed a number of engineered *H. volcanii* vectors and *H. volcanii* - *E. coli* shuttle vectors. For example, pWL102 is a 10.5 kb shuttle vector which carries 3.8 kb of pHV2, a 3.5 kb section encoding *H. volcanii* resistance to mevinolin (a competitive inhibitor of HMG Co-A reductase), and part of a pBR-derived plasmid including portions conferring ampicillin resistance in *E. coli*. This shuttle vector also carries a number of useful cloning sites. We have provided pWL102 to a number of investigators, including those in four labs in the ONR archaebacterial program. C. Daniels (Ohio State) has used pWL102 to construct an archaebacterial expression vector, which we will exploit in future work. Others have made vectors selectable and maintainable in *H. halobium*, using the mevinolin resistance determinant of pWL102.

The mevinolin-resistance determinant cloned in pWL102 has been sequenced. The mutation responsible for resistance lies within a 1.9 kb stretch of DNA with strong sequence similarity to eukaryotic HMG Co-A reductase genes -- as expected. The carboxy-terminal half of the predicted *H. volcanii* protein shares about 45% sequence similarity with rodent HMG Co-A reductase. The amino-terminal half diverges from eukaryotic sequences, where these latter are most divergent from each other. Unlike the eukaryotic HMG Co-A reductases, the amino-terminal half of the *H. volcanii* enzyme appears to lack membrane spanning domains -- not surprising since this archaebacterial enzyme is a soluble protein. Although we have not sequenced the wild-type halobacterial gene, we know that the mutation conferring mevinolin resistance disrupts an MluI site within the coding sequence. This is the first instance of a point mutation within an HMG Co-A reductase gene producing resistance to mevinolin. In eukaryotes, resistance most often results from gene amplification. We have now also found instances of amplification in *H. volcanii*. Amplified regions appear as very intense bands on gels of restricted genomic DNA. Although these regions vary in length between resistant strains, all contain the HMGCoA reductase-coding region identified by sequencing of the point mutation. Amplification is unstable in the absence of selection. A detailed molecular characterization of amplification mutants aimed at understanding the amplification process (also responsible for trimethoprim and pseudomonic acid resistance) will be undertaken in the next year.

While the 3.5 kb of homology with the *H. volcanii* genome carried by pWL102 has not presented any particular problems with stably maintaining self-replicating vectors, there is a low frequency of integration of the plasmid within the genomic HMGCo-A reductase gene region. We are interested

in minimizing vector integration. Of particular interest in this regard is a vector lacking the mevinolin-resistance determinant but bearing the *H. volcanii* homolog of the *E. coli trpB* gene, since we have also constructed an *H. volcanii* host strain in which the *trpB* gene has been deleted, and which therefore has no sequence homology with plasmid (see host development, below). Potential vectors which permit selection through complementation of auxotrophic mutations in other amino acid biosynthetic pathways, for instance those for leucine, arginine, and histidine (see below) have been produced easily by shotgun cloning wild-type DNA into pHV2, pWL102 and their derivatives, and could be similarly exploited. We have also constructed shuttle vectors which bear $tRNA^{Ser}$-derived amber, ochre or opal suppressors and carry only 248 bp of homology with the genome. We expect that these vectors will be selectable in *H. volcanii* nonsense mutants, but testing of these vectors awaits the results of screening our mutant collection for such nonsense mutant hosts (see below).

Host development

We originally favored development of *Haloferax volcanii* as a model halobacterial genetic system for four reasons: i) the relative stability of the *H. volcanii* genome, ii) the ability of *H. volcanii* to grow on minimal medium, iii) the existence of a mating system in this species, and iv) the availability of numerous auxotrophic mutants. The choice of *H. volcanii* as a model system has become even more easily justified as further genetic techniques such as transformation and cell fusion have been developed by us and M. Mevarech's laboratory in Tel Aviv, and as our contig map (and the construction of the ordered minimal set of cosmid clones on which it is based) nears completion.

Initially, we cured wild-type *H. volcanii* of its endogenous pHV2 to create an appropriate host for transformation with pHV2-derived vectors (98). This strain, WFD11, has served as the parent for virtually all of our subsequent strain constructions. EMS mutagenesis of WFD11 was followed with screening and identification of several hundred auxotrophs. These auxotrophs have, in turn, allowed screening by transformation with the minimal set of cosmid clones of the *H. volcanii* genome (mentioned above) to identify cosmids which carry complementing wild type genomic sequence.

Some of the complementing sequences have been extensively characterized (see below). These characterizations have lead to the ability to perform sophisticated host constructions. For example, the mevinolin resistance (mev^R) gene was cloned between sequences which flank the *H. volcanii trpB* gene, and WFD11 was transformed with this as a non-replicating circular construct. Selection for mev^R resulted in the recovery of mutants which had integrated the mev^R gene at *trpB* through a single crossover event. Subsequent resolution of the duplicated *trpB*-flanking sequences resulted in replacement of the *trpB* gene with the mev^R gene. Similarly, by transforming with a construct in which the mev^R gene is external to the *trpB*-flanking sequences, it is possible to recover *trpB* deletions in which the integrated sequence duplications are resolved through deletion of both the mev^R gene and the *trpB* gene from the *trpB* region. The construction of such strains is under way.

Applications

<u>Mapping.</u> The vector and transformation systems described above have been integrated into ongoing work in this laboratory to create a physical map of the *H. volcanii* genome using bottom-up mapping by ordering a set of cosmid clones and top-down mapping of restriction fragments by pulsed field gel electrophoresis (mapping work supported by Medical Research Council). By transforming the EMS-generated auxotrophic mutants mentioned above with a minimal set of about

160 *E. coli* cosmid clones of *H. volcanii* DNA, we have succeeded in mapping ~130 alleles to about 30 different clusters. These include alleles representing serine, isoleucine-valine, threonine, methionine, glutamine, early aromatic, tyrosine, phenalalanine, tryptophan, lysine, histidine, guanosine, pyrimidine, arginine and leucine biosynthetic pathways. This approach has also allowed us to determine the genetic locus for several auxotrophic alleles which remain unidentified. We have begun to look at several of the amino acid biosynthetic pathways in detail.

Histidine pathway. We have mapped 18 of the 25 histidine mutants in our collection to four chromosomal locations (i.e. mapped to non-overlapping cosmid clones). One of these map locations also complements the *his-1* allele of WR256 (from M. Mevarech's *H. volcanii* collection). In work done entirely by transforming WR256 with non-replicating, *E. coli*-cloned *H. volcanii* DNA or restriction fragments (i.e. integration required to replace the mutant allele), the complementing sequence was localized. Sequencing of 2 kb of DNA revealed a 1086 nt (361 aa) homolog to the *E. coli hisC* gene (encoding histidinol-phosphate aminotransferase). The encoded protein was found to be 32% identical to *E. coli*, 33% to *Bacillus subtilis* and 27% to *Saccharomyces cerevisiae*. There do not appear to be additional genes near the *hisC* gene as judged by examination of upstream and downstream sequence.

Arginine pathway. We have mapped 20 of our arginine mutants to one cosmid. This cosmid also complements the *arg-1* allele of WR256. Complementing DNA was localized as in the case with histidine and about 3 kb have been sequenced. We believe that there are five genes within the region which has been sequenced, including (based on sequence similarity with data in Genbank) *H. volcanii* homologs of the *E. coli* genes *argF*, *argB*, *pyrB* as well as the eukaryotic gene *oat* (ornithine aminotransferase). The gene which complements the *arg-1* allele of WR256 has not yet been identified, probably because sequence information available from Genbank on genes of the arginine biosynthetic pathway is incomplete. Sequencing is continuing both upstream and downstream.

Tryptophan mutants. We have mapped 29 of our tryptophan mutants to two unlinked cosmids. Based on growth of mutants on biosynthetic pathway intermediates, these 29 mutants represent, at minimum, five separate genes. Over 3 kb of one cluster has been sequenced and found to include *H. volcanii* homologs of the *E. coli trpC trpB* and *trpA* genes. The *trpA* enzyme has about 35% sequence similarity to *E. coli* or yeast enzymes; the *trpB* enzyme has about 50% sequence similarity to enzymes from *E. coli* or yeast.

Leucine pathway. About two dozen leucine auxotrophs have been characterized by growth on pathway intermediates into at least three classes. All of these map to one cosmid (~40 kb of *H. volcanii* DNA). Three kilobases of DNA from this cosmid has been cloned into the shuttle vector pWL102 and shown to complement several auxotrophs. This DNA is being sequenced.

Suppressor tRNAs. We are in the process of characterizing our collection of auxotrophs in others ways as well. Using site-directed mutagenesis we have changed the anticodon of a cloned *H. volcanii* $tRNA^{Ser}$ gene to be complementary to the three nonsense codons, thus creating potential suppressor tRNAs. A 248 bp sequence including the tRNA gene has been cloned into pWL102 for each of the suppressor tRNAs plus the wild type. Thus each plasmid is isogenic except for the bases encoding the anticodon. We are currently screening our collection of auxotrophs for nonsense mutants by transformation with the pooled suppressor tRNAs. Those auxotrophs which can be rescued by the plasmids will be re-transformed to determine if rescue is specific to one of the suppressor tRNAs. In collaboration with the laboratory of C. Daniels (Ohio State Univ.) we are also including in our screening a cloned *H. volcanii* $tRNA^{Trp}$ gene which has been altered to be a potential UGA reader. Once we've identified nonsense mutants we will try using these as hosts for shuttle vectors which only rely on the cloned suppressor tRNAs for selection (as mentioned above).

WORK PLANNED FOR YEAR THREE:

1) We have made several unsuccessful attempts to screen for restriction minus derivatives of WFD11. Although, to date, restriction has not prevented any of the work which we have attempted, it would still be useful to have a restriction minus strain. We will continue to pursue this goal and we expect that, given the increased sophistication of the genetic approaches available to us, we will be successful.

2) We will carry on with characterizing our mutant collection for nonsense mutants using the already constructed suppressor tRNA bearing vectors. Nonsense mutants will then serve as hosts for already-constructed vectors carrying only the small suppressor tRNA region of homology with the genome. Also we will use these hosts to determine the efficiency of recombination of short DNA sequences bearing suppressor tRNAs.

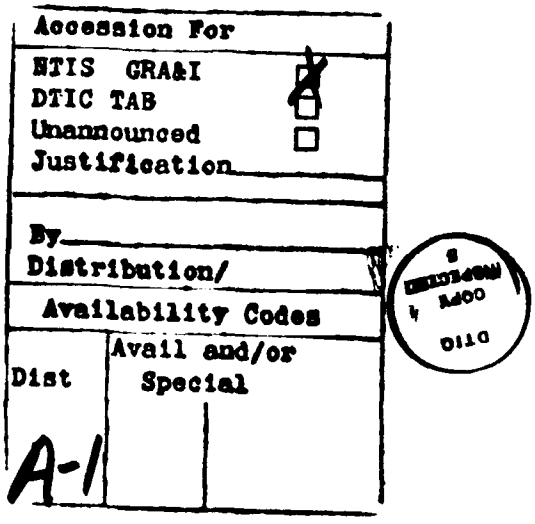
3) We will characterize our collection of auxotrophs for cold-sensitive and heat-sensitive mutants. Such conditional lethal mutants have been invaluable in the characterization of the molecular biology and genetics of eubacteria and eukaryotes.

4) We have two cosmids carrying overlapping *H. volcanii* clones (therefore <80 kb total *H. volcanii* DNA) which carry markers for *ilv*, *met*, *ser* and *his*. We will use this region to try to develop classical fine mapping techniques.

5) We will pursue the molecular genetics of tryptophan biosynthesis in *H. volcanii* in depth. This will involve completion of sequencing of the remaining biosynthetic genes and assaying gene expression at the protein and mRNA levels, in wild type and in mutants we suspect have altered regulation. Our preliminary work has involved several tryptophan analogs which easily inhibit growth, and mutants we have isolated which are resistant to these analogs. Some resistance determinants will probably also make good selectable markers for vector construction.

6) We will continue analyis of the HMGCoA reductase gene and mechanisms of resistance to mevinlolin through point mutation and amplification.

7) Two collaborative projects, formulated as a result of discussions at the Williamsburg meeting, will be carried out. The first involves Frank Robb (Maryland) -- and has us mapping and sequencing the *H. volcanii* glutamine synthetase gene while he characterizes mutants we have in this gene. The second collaboration is with John Spudich (Albert Einstein). He finds that, contrary to belief among halobacteriologists, *H. volcanii* is motile and photoresponsive. Spudich would like to refine the biochemical genetic dissection of the halobacterial sensory rhodopsin system he has begun so nicely with *H. halobium*. It should be possible to do parallel work in *H. volcanii*, as well as to develop tools for the genetics of *H. halobium*.



PUBLICATIONS:

We list only publications related to this project. Those marked with asterisks were submitted since ONR support began, and acknowledge this support. Reprints of articles published since our last annual report or preprints of articles recently submitted for publication are enclosed.

84. Cline, S.W. and Doolittle, W.F. (1987)
Efficient transfection of the archaebacterium *Halobacterium halobium*. J. Bacteriol. 169:1341-1344.

89. Charlebois, R.L., Lam, W.L., Cline, S.W. and Doolittle, W.F. (1987)
Characterization of pHV2 from *Halobacterium volcanii* and its use in demonstrating transformation of an archaebacterium. Proc. Natl. Acad. Sci. USA 84:8530-8534.

94. Charlebois, R.L. and Doolittle, W.F. (1989)
Transposable elements and genome structure in halobacteria. In Mobile DNA, M. Howe and D. Berg, eds. American Society for Microbiology, pp. 297-307.

95.* Cline, S.W., Lam, W.L., Charlebois, R.L., Schalkwyk, L.C. and Doolittle, W.F. (1989)
Transformation methods for halophilic archaebacteria. Can. J. Microbiol. 35:148-152.

96.* Charlebois, R.L., Hofman, J.D., Schalkwyk, L.C., Lam, W.L. and Doolittle, W.F. (1989) Genome mapping in halobacteria. Can. J. Microbiol. 35:21-29.

98.* Lam, W.L. and Doolittle, W.F. (1989)
Shuttle vectors for the archaebacterium *Halobacterium volcanii*. Proc. Natl. Acad. Sci. USA 86:5478-5482.

99.* Cline, S.W., Schalkwyk, L.C., and Doolittle, W.F. (1989)
Transformation of the archaebacterium *Halobacterium volcanii* with genomic DNA. J. Bacteriol. 171:4987-4991.

100.* Lam, W.L., Charlebois, R.L. and Doolittle, W.F. (1980)
Progress in the molecular biology of the archaebacteria. UCLA Symp. Mol. Cell. Biol. (New Series), *in press*.

101.* Doolittle, W.F., Charlebois, R.L., Schalkwyk, L.C., Lam, W.L., Conover, R.K., Tsouluhas, D., Cline, S.W., Hofman, J.D. and Cohen, A. (1989)
Physical mapping and gene transfer methods for *Halobacterium (Haloferax) volcanii*. Proceedings of FEMS Workshop on Halophilic Bacteria, F. Rodriguez-Valera, ed., Plenum, *in press*.

102.* Conover, R.K. and Doolittle, W.F. (1990)
Characterization of a gene involved in histidine biosynthesis in *Halobacterium (Haloferax) volcanii*: isolation and rapid mapping by transformation of an auxotroph with cosmid DNA. *Submitted* to J. Bact. in December, 1989.

103.* Schalkwyk, L.C., Charlebois, R.L. and Doolittle, W.F. (1990)
Insertions sequences on plasmid pHv1 of *Haloferax (Halobacterium) volcanii*. *Submitted* to J. Bacteriol. in December, 1989.

REPORTS:

Aspects of this work have been presented at:

Third International Symposium on the Molecular Biology of the Archaebacteria, Victoria, British Columbia, 1989.

UCLA Symposium on Molecular Evolution, Lake Tahoe, California, 1989.

Canadian Institute for Advanced Research Genome Evolution Meeting, Victoria, British Columbia, 1989.

Molecular Evolution Course, Wood's Hole, Massachusetts, 1989.

FEMS-NATO Advanced Research Workshop on General & Applied Aspects of Halophilic Microorganisms, Alicante, Spain,1989.

Office of Naval Research Archaebacteria Meeting, Williamsburg, Virginia, 1989.

RNA Relics Meeting, University of California, San Francisco, 1989.

Annual Tryptophan Meeting, Asilomar, California, 1990.

PERSONNEL SUPPORT:

This grant pays the salary of a senior technician, Steven Cline. It contributes, to various extents, to the research expenses (but not salaries or stipends) of Wan Lam, Leo Schalkwyk (graduate students), Annalee Cohen (a technician), and Rob Charlebois, Keith Conover and DeCheng Yang (postdocs). Yang is a Chinese citizen.